Flow of information in the light-triggered cyclic nucleotide cascade of vision

(rhodopsin/transducin/cyclic GMP phosphodiesterase/guanyl nucleotides/signal amplification)

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ABSTRACT   Photolyzed rhodopsin catalyzes the exchange of GTP for GDP bound to a protein in retinal rod outer segments. We previously proposed that the GTP complex of this protein regulates the cyclic GMP phosphodiesterase and that it may be the first amplified intermediate in visual excitation [Fung, B. K.-K. & Stryer, L. (1980) Proc. Natl. Acad. Sci. USA 77, 2500–2504]. We report here the identification and characterization of transducin, a regulatory protein consisting of three kinds of polypeptide chains: T_α (39 kilodaltons), T_β (36 kilodaltons), and T_γ ($\approx$10 kilodaltons). Reconstituted membranes containing transducin and rhodopsin but no phosphodiesterase exhibit GTPase activity and amplified binding of guanosine 5'-[β,γ-imido]triphosphate (p[NH]ppG), a nonhydrolyzable analog of GTP, on illumination. A single photolyzed rhodopsin molecule led to the uptake of p[NH]ppG by 71 molecules of transducin. High-pressure liquid chromatography showed that the binding site for GTP is on the α subunit of transducin. The isolation of the complex of p[NH]ppG with T_α enabled us to determine whether this species is the activator of the phosphodiesterase. We found that phosphodiesterase on unilluminated disc membranes can indeed be fully activated by addition of T_α containing bound p[NH]ppG. These findings strongly suggest that transducin is the first amplified information-carrying intermediate in the cyclic nucleotide cascade of vision.

Cyclic GMP appears to be important in visual excitation (1–3). Electrophysiological studies have shown that cyclic GMP depolarizes the plasma membrane of rod outer segments (ROS) within milliseconds after being injected intracellularly and that it increases the latency of the light-induced hyperpolarization (4). A single photolyzed rhodopsin molecule can lead to the hydrolysis of more than 10^5 molecules of cyclic GMP (5, 6). This large gain is achieved in two stages (7): the first is the activation of hundreds of phosphodiesterase molecules (5), and the second is the hydrolysis of about a thousand cyclic GMP molecules per sec by each activated phosphodiesterase (8, 9). Photolyzed rhodopsin catalyzes the exchange of GTP (or guanosine 5'-[β,γ-imido]triphosphate) (p[NH]ppG), a nonhydrolyzable analog of GTP) for GDP bound to disc membranes (10, 11). We observed a high degree of amplification ($\approx$500) in this exchange and proposed that the GTP complex of a regulatory protein activates the phosphodiesterase whereas the GDP complex does not (11). We report here experiments designed to test the hypothesis (11) that the flow of information in the cyclic nucleotide cascade is

$$R^* \longrightarrow T\text{–GTP} \longrightarrow PDE^*$$
$$1 \qquad \approx 500 \qquad \approx 500$$
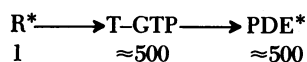
in which R* denotes photolyzed rhodopsin, T–GTP denotes the GTP complex of the regulatory protein, and PDE* denotes the active form of the phosphodiesterase. This scheme predicts that T–GTP can be formed in vitro in the absence of phosphodies-

terase and that the phosphodiesterase can be activated by T–GTP in the absence of photolyzed rhodopsin. In fact, both predictions are met, showing that T–GTP is likely to be the primary amplified information-carrying intermediate in the cyclic nucleotide cascade of vision. Consequently, we propose that this regulatory protein be called transducin.

Materials. Frozen bovine retinas were purchased from American Stores Packing (Lincoln, NE). The TSK-125 gel filtration column was obtained from Bio-Rad, hexylagarose from Miles, [^{3}H]p[NH]ppG, cyclic [^{3}H]GMP, and 5'-[^{14}C]GMP from Amersham, and [γ-^{32}P]GTP from New England Nuclear. Snake venom (Ophiophagus hannah) was obtained from Sigma. Phosphatidylcholine was purified from egg yolks (12). ROS were isolated as described (11), except that the 35% (wt/vol) sucrose flotation step was carried out only once to minimize losses of the phosphodiesterase and transducin. Reconstituted membranes containing hydroxylapatite-purified rhodopsin and phosphatidylcholine at a molar ratio of 1:125 were prepared by dialysis (13). These membranes were devoid of phosphodiesterase and transducin.

Buffer A (extraction buffer) consisted of 5 mM Tris·HCl, 0.5 mM MgCl$_2$, 0.1 mM phenylmethylsulfonyl fluoride (PhMeSO$_2$F), and 1 mM dithiothreitol, at pH 7.5. Buffer B (hexylagarose chromatography buffer) was 10 mM 4-morpholinepropanesulfonic acid (Mops), 2 mM MgCl$_2$, 0.1 mM PhMeSO$_2$F, and 1 mM dithiothreitol, at pH 7.5. Buffer C (high-pressure liquid chromatography buffer) was 20 mM Mops, 100 mM Na$_2$SO$_4$, 1 mM dithiothreitol, 0.1 mM PhMeSO$_2$F, and 1.5 mM MgCl$_2$, at pH 7.2. Buffer D (reconstitution assay buffer) was 60 mM KCl, 30 mM NaCl, 2 mM MgCl$_2$, 10 mM Mops, 1 mM dithiothreitol, and 0.1 mM PhMeSO$_2$F, at pH 7.5.

Assays. Protein concentrations were determined by the Coomassie blue binding method (14), with bovine serum albumin as a standard. NaDodSO$_4$/polyacrylamide gel electrophoresis was performed with a 10–18% polyacrylamide gradient (15).

GTPase activity was assayed by measuring the formation of radioactive orthophosphate (16). The assay mixture contained 10 μM [γ-^{32}P]GTP, 300 mM NaCl, and 10 μM photolyzed rhodopsin (in reconstituted membranes) in buffer B. A 20-μl aliquot of each column fraction was added to 20 μl of this assay mixture. After a 5-min incubation at 23°C, the reaction was stopped by addition of perchloric acid containing potassium phosphate; orthophosphate was then precipitated by addition of ammonium molybdate. The orthophosphate precipitate was collected by filtration and washed six times, and the radioactivity retained by

the filter was measured (16). The recovery of orthophosphate was about 85%.

The binding of [³H]p[NH]ppG to protein was measured by incubating 20 μl of each column fraction with 20 μl of buffer B containing 5 μM [³H]p[NH]ppG (containing ≈50,000 dpm), 2.5 μM photolyzed rhodopsin in reconstituted membranes, and 300 mM NaCl. After a 1-hr incubation at 4°C, 2 ml of ice-cold buffer B containing 10 mM NaCl was added, and the mixture was immediately filtered through a 0.45-μm type HA Millipore filter. This filter retained nucleotide bound to protein but not free nucleotide. The filter was washed three times with 2 ml of this buffer and its radioactivity was measured. The recovery of bound nucleotide was about 65%.

Phosphodiesterase activity was assayed by a modification of a described method (17). Samples were assayed in 0.1 ml of buffer C containing 2 mM cyclic GMP, cyclic [³H]GMP (250,000 dpm), and 5'-[¹⁴C]GMP (10,000 dpm). 5'-[¹⁴C]GMP served to monitor the recovery of the 5'-[³H]GMP product of the phosphodiesterase reaction. Each assay was initiated by addition of cyclic GMP. The sample was incubated on ice for 2 min, and the reaction was terminated by adding 0.1 ml of 20 mM nonradioactive cyclic GMP and boiling the mixture for 2 min. The 5'-[³H]GMP product was converted to [³H]guanosine by addition of 0.5 ml of snake venom (0.2 mg/ml) and incubation at 30°C for 15 min. This mixture was applied to a 0.7 × 2 cm DEAE-Sephadex column, and the [³H]guanosine was eluted with 2 ml of distilled H₂O. ³H and ¹⁴C were measured in the eluant.

RESULTS

Isolation of Transducin. Transducin extracted from ROS by Kühn's procedure (18) was further purified and concentrated by
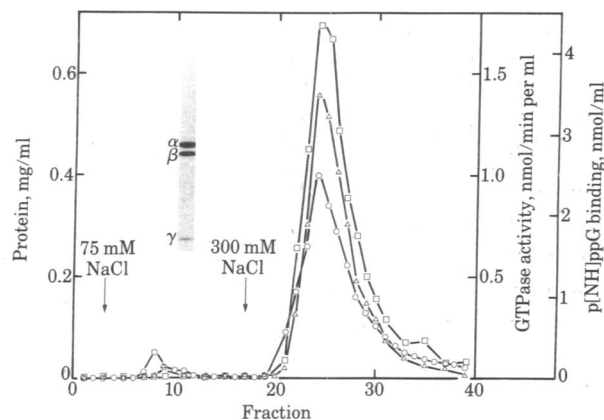

FIG. 1. Hexylagarose chromatography of transducin. A protein fraction containing transducin was extracted from ROS by a modification of Kühn's procedure (18). ROS from 200 retinas were suspended in 80 ml of ice-cold buffer A and immediately exposed to room light for 10 min. The bleached membranes were then sedimented at 45,000 × g for 30 min. The pellet was washed with buffer A four more times to remove all of the phosphodiesterase. Transducin bound to these membranes was then eluted by 50 ml of buffer A containing 0.1 mM GTP. The membrane suspension was centrifuged at 45,000 × g for 30 min, and the supernatant was collected. This extraction was repeated twice. The combined supernatants, containing 8 mg of protein, were centrifuged at 45,000 × g for 30 min to remove a small amount of residual ROS membranes. This solution was placed onto a 15 × 0.7 cm diameter column of hexylagarose. The flow rate was 20 ml/hr. The column was washed with buffer B and protein was then eluted by the stepwise addition of 75 mM NaCl and then 300 mM NaCl. About 5.5 mg of protein was recovered in the 300 mM NaCl fractions. ○, Protein concentration; △, GTPase activity; □, p[NH]ppG binding capacity. (*Inset*) NaDodSO₄/polyacrylamide gel pattern of the peak fraction.
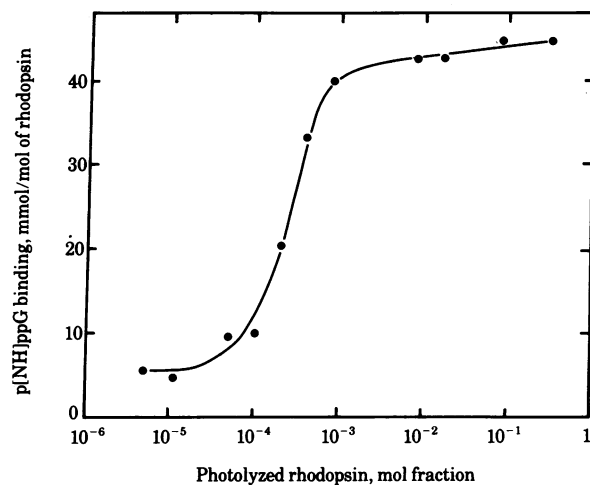

FIG. 2. Photolyzed rhodopsin in reconstituted membranes catalyzes the binding of p[NH]ppG to purified transducin. A 0.4-ml aliquot of transducin (1.2 mg/ml) was incubated with 0.8 ml of reconstituted membranes containing 75 μM rhodopsin. After 12 hr in the dark at 4°C, the membranes were pelleted at 45,000 × g for 30 min. About 73% of the transducin was bound to the membranes. The membrane pellet was resuspended in buffer D to a final rhodopsin concentration of 3 μM and then divided into 0.3-ml aliquots. An equal volume of 20 μM [³H]p[NH]ppG (containing 600,000 dpm) was then added to each aliquot and the mixtures were immediately subjected to different degrees of photolysis. After 30 min at 22°C, the amount of tritiated nucleotide bound to transducin was assayed as described (8).

chromatography on hexylagarose (Fig. 1). This step also removed unbound guanyl nucleotides. A small amount of protein impurity was eluted by 75 mM NaCl. An increase in salt concentration to 300 mM led to the elution of a protein fraction with GTPase activity and p[NH]ppG-binding (or GDP-binding) activity when assayed in the presence of reconstituted membranes containing photolyzed rhodopsin. NaDodSO₄/polyacrylamide gel electrophoresis (Fig. 1 *Inset*) showed that these fractions contain three polypeptides, which we designate T$_\alpha$ (39 kilodaltons), T$_\beta$ (36 kilodaltons), and T$_\gamma$ (≈10 kilodaltons). These polypeptides were similar to those found previously (18, 19). Sucrose density gradient sedimentation experiments (unpublished results) suggested that T$_\alpha$, T$_\beta$, and T$_\gamma$ form a complex in the absence of p[NH]ppG, whereas T$_\alpha$ is dissociated from T$_\beta$ and T$_\gamma$ when this nucleotide is bound. This change in the state of association of the complex proved useful in the purification of T$_\alpha$ (see below).

Amplified Binding of p[NH]ppG to Transducin in Reconstituted Membranes. We previously reported that photolyzed rhodopsin catalyzes the exchange of GTP (or p[NH]ppG) for GDP bound to ROS membranes (11). The experiment shown in Fig. 1 demonstrates that transducin can bind p[NH]ppG. The degree of phosphodiesterase contamination in our purified transducin preparation was less than 1% on a molar basis. The availability of purified transducin enabled us to determine whether the *amplified* binding of p[NH]ppG to transducin can occur in the absence of phosphodiesterase. Purified transducin was incubated in the dark with reconstituted membranes containing hydroxylapatite column-purified rhodopsin and phosphatidylcholine. About 73% of the added transducin became bound to this membrane. The stoichiometry under these conditions (see Fig. 2 legend) was 1 molecule of transducin per 17 molecules of rhodopsin. When subjected to different degrees of photolysis, this reconstituted membrane system, like native ROS membranes (11), exhibited amplified uptake of p[NH]ppG (Fig. 2). The amount of nucleotide bound was half-maximal when the degree of photolysis was 0.027%, which corresponds
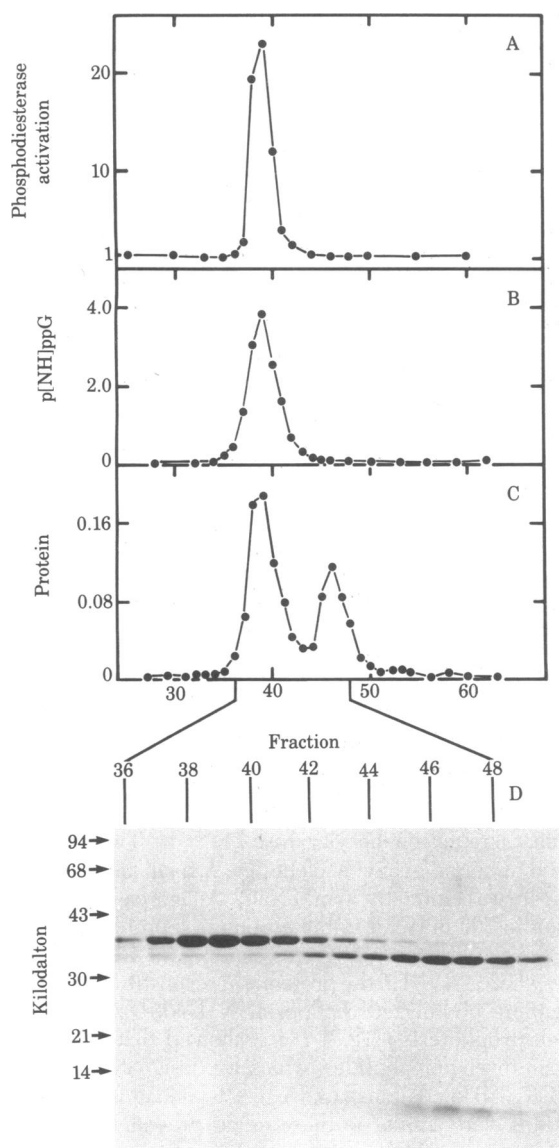
FIG. 3. High-pressure liquid chromatography of transducin. The complex of this protein with p[NH]ppG was prepared by incubating purified transducin (1 mg/ml) in 4 ml of buffer B containing 300 mM NaCl with 0.15 mM [³H]p[NH]ppG and 5 μM photolyzed rhodopsin in reconstituted membranes for 12 hr on ice. The reconstituted membranes were removed by centrifugation at 45,000 × g for 15 min. The supernatant was diluted with 36 ml of buffer B and applied to a 10 × 0.7 cm hexylagarose column. The flow rate was 10 ml/hr. Unbound p[NH]ppG was removed by washing the column with buffer B. Fractions containing p[NH]ppG that was bound to protein eluted with 300 mM NaCl in buffer B were pooled and concentrated. This concentrated solution [0.3 ml of protein (1.4 mg/ml)] was placed onto a 30 × 0.75 cm TSK-125 (Bio-Rad) gel filtration column equilibrated with buffer C. The pressure was 100 lb/inch² (4.22 × 10⁵ Pa); the flow rate was 0.2 ml/min. The void volume (determined with thyroglobulin) corresponded to fraction 27; the included volume (determined with phenylalanine) corresponded to fraction 64. The fraction volume was 0.22 ml. (A) Stimulation of phosphodiesterase activity of unilluminated ROS membranes containing 0.2 nmol of rhodopsin by addition of a 10-μl aliquot of a column fraction. The basal enzyme activity (0.6 nmol of cyclic GMP hydrolyzed per min) is denoted by a level of 1 on the ordinate. (B) p[NH]ppG concentration, nmol/ml. (C) Protein concentration, mg/ml. (D) NaDodSO₄/polyacrylamide gel electrophoresis pattern of the peak column fractions.

to the catalyzed uptake of 71 p[NH]ppG molecules per photolyzed rhodopsin molecule. This result strongly suggests that the phosphodiesterase is not required for the catalyzed uptake of

p[NH]ppG. In native disc membranes, the degree of amplification was 500 under similar experimental conditions, and the uptake was shown to correspond to an exchange of p[NH]ppG for GDP (11). Several factors may account for the lower amplification observed with reconstituted membranes. Only half of the photolyzed rhodopsin molecules are properly oriented in the reconstituted membrane. Furthermore, the reconstituted vesicles are severalfold smaller than the native ones and their lipid composition is different.

p[NH]ppG Binds to the α Subunit of Transducin. The subunit of transducin containing the guanyl nucleotide-binding site was identified by its tight binding of radioactive p[NH]ppG. Transducin was incubated with radioactive p[NH]ppG in the presence of photolyzed reconstituted membranes and excess nucleotide was removed by hexylagarose chromatography. The transducin–nucleotide complex was fractionated by high-pressure liquid chromatography. The elution profile exhibited two protein peaks (Fig. 3C). The NaDodSO₄/polyacrylamide gel electrophoresis pattern of these fractions (Fig. 3D) showed that the first peak contains T_α whereas the second contains T_β and T_γ. The radioactive p[NH]ppG eluted together with T_α. A separate experiment showed that free nucleotide elutes in fractions 54–56, some 15 fractions after T_α. In particular, the absence of detectable free nucleotide in the fractions after those containing T_α showed that the complex of p[NH]ppG with T_α is very tight. The number of p[NH]ppG bound to T_α was 0.8 ± 0.3 (SEM).

One or more of the subunits of transducin probably interacts with the matrix of the high-pressure liquid chromatographic column because their separation was greater than expected on the basis of gel filtration alone. The separation of T_α from T_β and T_γ by high-pressure liquid chromatography was much better when p[NH]ppG was bound than in its absence. In particular, it was not feasible to obtain T_α free of T_β and T_γ. The simplest interpretation is that T_α is dissociated from T_β and T_γ when
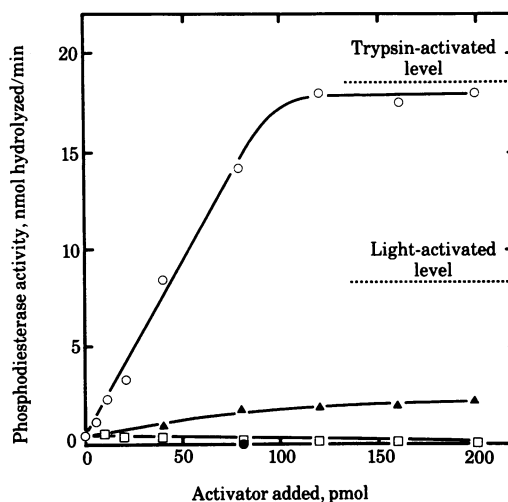

FIG. 4. Activation of phosphodiesterase bound to unilluminated ROS membranes by T_α–p[NH]ppG. Phosphodiesterase in unilluminated ROS membranes (containing 0.2 nmol of rhodopsin) was incubated with T_α–p[NH]ppG (○), p[NH]ppG alone (▲), or transducin–GDP (□) in 80 μl of buffer C. T_α–p[NH]ppG without added membranes (●) contained almost no detectable phosphodiesterase activity. After 10 min on ice, phosphodiesterase activity was measured. Maximal phosphodiesterase activity (upper dashed line) was obtained by incubating unilluminated ROS membranes (containing 0.2 nmol of rhodopsin) with 10 ng of N-tosylphenylalanine chloromethyl ketone-treated trypsin in the same buffer for 5 min. The light-activated phosphodiesterase level (lower dashed line) was obtained by bleaching the membranes in the presence of 10 μM p[NH]ppG. The concentration of T_α–p[NH]ppG was determined from its content of radioactive nucleotide.

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p[NH]ppG is bound. Sucrose density gradient centrifugation profiles (unpublished results) are consistent with this interpretation.

Phosphodiesterase Bound to Unilluminated ROS Membranes Can Be Fully Activated by T_α–p[NH]ppG. Phosphodiesterase bound to unilluminated membranes was activated by fractions containing p[NH]ppG bound to T_α (first peak of Fig. 3C). In contrast, fractions containing T_β and T_γ (second peak in Fig. 3C) did not activate the phophodiesterase. Previous studies showed that the phophodiesterase could be activated by photolyzed rhodopsin in the presence of GTP (or p[NH]ppG) or by the addition of a small amount of trypsin (8). The catalytic activity of trypsin-activated phosphodiesterase was severalfold higher than that of light-activated phosphodiesterase. How does the level of phosphodiesterase activated by the addition of T_α–p[NH]ppG compare with the levels obtained by activation with light or trypsin? As shown in Fig. 4, the activity of phosphodiesterase bound to unilluminated ROS membranes increased nearly linearly with the amount of T_α–p[NH]ppG added up to an asymptotic value. This limiting value was 2.2-fold as high as the catalytic activity obtained by activation with light in the presence of p[NH]ppG. Most striking, the maximal value attainable by the addition of T_α–p[NH]ppG was nearly the same as that obtained by activation of the phosphodiesterase with trypsin. Very little activation was observed on addition of p[NH]ppG alone or of the transducin complex without this nucleotide (Fig. 4).

DISCUSSION

How does a single photolyzed rhodopsin molecule activate several hundred phosphodiesterase molecules? Our experiments indicate that this activation occurs in two stages. First, transducin–GTP is formed in an exchange reaction that is catalyzed by photolyzed rhodopsin. Then, transducin–GTP switches on the phosphodiesterase. The first reaction can occur in the absence of photolyzed rhodopsin. Hence, information flows from photolyzed rhodopsin to transducin–GTP and then to the phosphodiesterase. A reaction scheme based on these findings is shown in Fig. 5. Amplification is achieved in the first part of the cycle in going from transducin–GDP to transducin–GTP. Several hundred transducin–GTP molecules are formed per photolyzed rhodopsin molecule (11). Binding studies (18) have

shown that transducin–GDP has high affinity and that transducin–GTP has low affinity for photolyzed rhodopsin (R*), which would enable R* to recycle rapidly and catalyze many exchanges of GTP for bound GDP. Conversely, it seems likely that transducin–GTP has high affinity and that transducin–GDP has low affinity for the phosphodiesterase, which would enhance its capacity to serve as an excitation carrier after illumination. The GTPase activity of transducin is an essential part of the deactivation mechanism that returns the system to the dark state. The cycle proposed here is reminiscent of the elongation factor Tu–Ts cycle in protein synthesis, which is also driven by the hydrolysis of GTP (20). The affinities of transducin and elongation factor Tu for other proteins depend on whether GTP or GDP is bound to them. An even closer analogy, perhaps even a homology, is provided by the mechanism of activation of adenylate cyclase. A guanyl nucleotide-binding protein has been shown to be the information-carrying intermediate between a hormone receptor and adenylate cyclase (21).

Our experiments raise several questions concerning the roles of the β and γ subunits of transducin. First, are they required for the catalyzed uptake of GTP by the α subunit of transducin? Second, do the β and γ subunits contribute to the GTPase activity of the transducin complex? A direct way of answering these questions would be to isolate the complex of the α subunit with either GTP or GDP and determine the effects on its properties of adding the β and γ subunits. However, p[NH]ppG bound to the α subunit cannot readily be displaced by GTP or GDP, and so this experiment is not yet feasible. It may be necessary to prepare T_α–GDP in a different way. Third, are the β and γ subunits required in addition to T_α–GTP for the activation of the phosphodiesterase? The experiment shown in Fig. 4 indicates that T_α–GTP is the information-carrying species but it does not rule out the possibility that T_β and T_γ also participate in the activation process. A small amount of these subunits was present in the unilluminated membranes used to assay the activation of the phosphodiesterase. This question could be answered by preparing a reconstituted system free of T_β and T_γ. It will also be interesting to learn how T_α–GTP activates the phosphodiesterase. The activation of this enzyme by the limited proteolytic action of trypsin suggests that the intact phosphodiesterase is subject to an inhibitory constraint (8, 19). The maximal enzymatic activity attainable by the addition of T_α–p[NH]ppG is nearly the same as that produced by tryptic digestion (Fig. 4). The simplest interpretation is that the addition of this species (or of T_α–GTP) removes the same inhibitory constraint as does the proteolytic action of trypsin. T_α–GTP might bind an inhibitory subunit of the phosphodiesterase or it might compete with such a subunit for binding to the phosphodiesterase. Alternatively, T_α–GTP might activate the phosphodiesterase by binding to the enzyme–inhibitor complex and altering the interaction of the inhibitory subunit with the catalytic subunit of this enzyme.
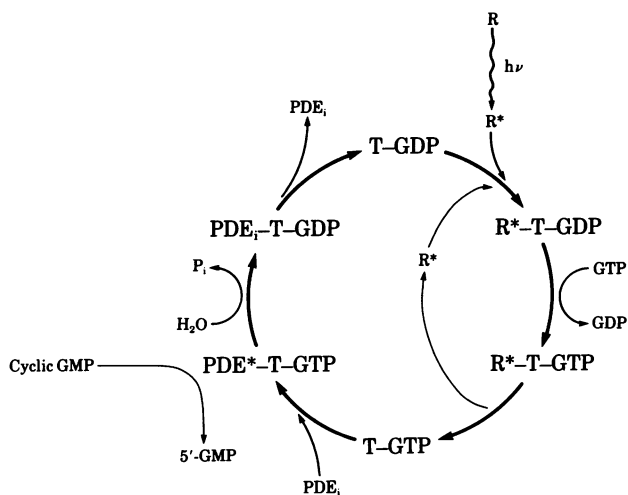
FIG. 5. Proposed scheme for the flow of information in the light-activated cyclic nucleotide cascade of vision. PDE, phosphodiesterase; T, transducin; R*, photolyzed rhodopsin.

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